IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#20/K.T. 6/13 Response

In re application of	:	**ATTN: BOX RCE**
Minoru FUJIMORI et al.	:	Docket No. 2001_0206A
Serial No. 09/816,391	:	Group Art Unit 1635
Filed March 26, 2001	:	Examiner Brian A. Whiteman
ANAEROBIC BACTERIUM AS A DRUG FOR CANCER GENE THERAPY		**Confirmation No. 7242**

PRELIMINARY REMARKS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is responsive to the Advisory Action issued May 15, 2003. Favorable consideration of the pending claims is respectfully requested.

Applicants respectfully request a continued examination (RCE) for the present application, entry of the amendments filed May 2, 2003, and a withdrawal of the finality of this application. Favorable reconsideration is respectfully requested in view of the claim amendments dated May 2, 2003, and the following remarks.

In the Advisory Action, the Examiner rejects the claims in the present application roughly for the following reasons:

(1) Failure to meet the enablement requirements

(2) Lack of novelty and inventive step

Reason (1) is one of the reasons of rejection stated in the Final Office Action. As to the reason of rejection, Applicants point out:

(A) There are patents, wherein a "genus" as a taxonomy is claimed without detailed description in the examples, by pointing out the existence of US 5,601,999, and

(B) By pointing out the existence of Li et al. (Cancer Gene Therapy, 2003;10:105-111), the claimed invention in the present application can be carried out using *Bifidobacterium* species other than *Bifidobacterium longum* based on the disclosure of the present application together with the knowledge that people skilled in the art have.

The Examiner states:

"[t]he US Patent 5,601,999 cited in the argument is directed to an anti-tumor agent and the disclosure of the Patent does not use the same methods or materials as set forth in the claimed invention", and

"the post-filing reference (Li, Cancer Gene Therapy, 2003:10:105-111) cited in the argument displaying that one species of the claimed species of *Bifidobacterium* can be used in the claimed invention does not overcome the unpatentability of efficiently transforming the other claimed *Bifidobacterium* species".

Applicants respectfully request reconsideration of the Examiner's position.

(1) On the failure to meet the enablement requirements

The Examiner states that the US patent is directed to an anti-tumor agent and the disclosure of the Patent does not use the same methods or materials as set forth in the claimed invention. Applicants' intention to submit the US Patent is to show that there is a US patent wherein only some of the species belonging to a genus are disclosed in the examples but claims on the genus are patented, but not to show that the US Patent should be related to the present application in any technological sense. The US patent was submitted to show that the Examiner's statement that the claimed inventions should be disclosed in the examples is not appropriate from the practice viewpoint of US patent examination. Applicants respectfully ask the Examiner to review a copy of the attached US patents (4,519,913, 6,569,653, and 6,217,766), the existence of which clearly indicates that the above-mentioned practice is widespread. Applicants kindly request for a fair

and sincere examination of the present application, and believe that such an examination will be carried out by the Examiner in the USPTO, where fairness and sincerity are highly valued.

If the Examiner finds any reasonable reasons of rejection in distinguishing the present case and other cases regarding the applicability of the above-mentioned practice, Applicants respectively reconsider the present application waiting appropriate comments from the Examiner.

It is evident from the existence of Li et al. that the claimed inventions can be carried out using other species from the *genus Bifidobacterium* than *Bifidobacterium longum* or *Bifidobacterium adolescentis,* based on the disclosure in the present application together with the knowledge that people skilled in the art have.

Li et al. teach that *Bifidobacterium adolescentis* is useful as a gene delivery vehicle. Li et al. also teach that *Bifidobacterium bifidum* and *Bifidobacterium longum* are useful as a gene delivery vehicle, since both can selectively germinate and grow in the hypoxic regions of solid tumors after intravenous injection. See page 105, right column, lines 4-6, citing two publications as footnotes 8 and 9.

Li et al. further teach that there are several advantages of selecting species of *Bifidobacterium* as a gene delivery vector for cancer gene therapy. See page 110, the paragraph bridging left and right columns, and right column, second complete paragraph.

The claims are limited to a bacterium selected from *Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium pseudolongum, Bifidobacterium thermophirum, Bifidobacterium breve* and *Bifidobacterium infantis.*

The teachings of the specification and Li et al. establish that three of the seven claimed species, *Bifidobacterium adolescentis, Bifidobacterium bifidum* and *Bifidobacterium longum,* are useful as a gene delivery vehicle.

Based upon these teachings and the similarities of species of *Bifidobacterium,* one skilled in the art would have a reasonable expectation that the other four claimed species, *Bifidobacterium pseudolongum, Bifidobacterium thermophirum, Bifidobacterium breve* and *Bifidobacterium infantis* would also be useful as a gene delivery vehicle.

Therefore, it is respectfully submitted that one skilled in the art would be capable of practicing the invention according to the claims with a reasonable expectation of success and without undue experimentation. Accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

(2) On the rejection based on the lack of novelty and inventive step

As to the rejections of novelty and inventive step of the present application, it is stated by the Examiner as follows:

"The Declaration filed on 5/2/03 under 37 CFR 1.131 has been considered but is ineffective to overcome the 102(a) rejection as anticipated by Yazawa et al. (claims 3, 8, and 9) or the 102(a) rejection as anticipated by Babinocova (claim 3, 4, 8, and 9) or the 103(a) rejection for claims 3-5, 8, 9, 12, 14, 16, 19, 21, 24, and 28-31 as being unpatentable over Babincova taken with Tagliabue.

The Declaration only establishes that the Yazawa et al. article is a 102(a) reference. The Yazawa publication has a different inventive entity then the entity in the instance application because the Yazawa's publication lists five of the seven inventors listed in the application. The Office assumes that all of the inventors, absence evidence to the contrary, are the inventors of the application. The Declaration has not provided evidence that the seven inventors reduce the invention to practice before the five inventors listed on the Yazawa publication"

Applicants do not follow the position taken by the Examiner, stating "Yazawa's publication lists five of the seven inventors listed in the application" and "The Office assumes that all of the inventors, absence evidence to the contrary, are the inventors of the application."

The past procedure of the present application is rather straightforward, and is an example of typical applications in the sense that the application was filed based on the inventions disclosed in Yazawa et al. with additional inventions made by the rest of the inventors not listed in Yazawa et al.'s publication. That is, the present application is an example of patent applications wherein further elements of inventions are added at the time of filing. The inventions made by the five

authors listed in Yazawa's publication should receive a benefit of grace period. It would be quite a disadvantage if the Applicants are not able to receive a benefit of the grace period.

If there is any additional evidence which the Examiner may suggest, such as an additional Declaration of the five inventors/authors, the two inventors/non-authors, or the seven inventors, the Applicants would appreciate the Examiner's suggestion in this regard.

The Applicants appreciate the Examiner's indication that the claims would be allowable if limited to *Bifidobacterium longum* and *Bifidobacterium adolescentis*. However, such a reduction from the *genus Bifidobacteria* to the two species of *Bifidobacteria* leads to a patent that does not cover other *Bifidobacteria*. That is, anyone can make or use the inventions using *Bifidobacteria* not claimed in the present application, and will be meaningless from the economical point of view. That is, imposing too severe enablement requirements allow a third person to make and use the inventions, and such requirements are severe for the inventors completing the inventions with all efforts or the Applicants of the inventions. Further, it should be concluded that the situation where anyone can "steal" the inventions by making or using them lacks the fairness, not only in the United States, where fairness and equality are highly valued but in other countries or states.

The system of patent will be construed as having a purpose of contributing to the development of industries by providing a patent as an exclusive right for the cost of opening an invention. However, inventors would lose motivations and the industries will lose power if patent rights are not provided in an appropriate manner. That is, too severe requirements would not only be unbeneficial for inventors, but also be one of the indirect factors that would lose the benefits for the United States. For the patent system in the United States and its practice to continue to be fair and reliable, it is respectfully requested that the present application will be examined fairly and sincerely with the view of the ultimate purpose of the patent system.

Applicants have also noted the Examiner's indication that claim 32 is objected to as being dependent upon a rejected base claim 28. However please note that claim 32 is now in independent form after entry of the May 2, 2003 amendments.

Applicants may take appropriate responses with some considerations on the claims currently not subject to examination if the claims that are currently subject to examination are

regarded as patentable. Applicants may also take necessary responses with some considerations on formalities if the claims that are currently subject to examination are regarded as patentable. Accordingly, favorable consideration of the pending claims is respectfully requested.

Should the resolution of any minor issues place the application in condition for allowance, the Examiner is kindly invited to call the undersigned at the designated telephone number.

Respectfully submitted,

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Bifidobacterium adolescentis as a delivery system of endostatin for cancer gene therapy: Selective inhibitor of angiogenesis and hypoxic tumor growth

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In order to overcome difficulties that hampered widespread application of antiangiogenesis in cancer therapy, a highly specific delivery system may be engaged *in vivo* to deliver and express antiangiogenic genes. We selected a strain of *Bifidobacterium adolescentis* (*B. adolescentis*) as the delivery system to transport endostatin gene to solid tumors. *B. adolescentis* with endostatin gene were injected into tumor-bearing mice through the tail vein. After the mice were sacrificed, the tumor and some normal tissues of the mice were examined. *B. adolescentis* were only found in the tumors and no bacilli were found in other normal tissues. Also, a strong inhibition of angiogenesis had been shown to inhibit local tumor growth in the administrated group. These results suggested that *B. adolescentis* only germinated and proliferated in solid tumors and might be a highly specific and efficient vector for transporting anticancer genes into target tumor in cancer gene therapy.
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Keywords: **Bifidobacterium adolescentis*; endostatin; angiogenesis; tumor; gene therapy***

Angiogenesis is required for tumor progression and metastasis. In tumor growth process, tumor cells promote angiogenesis by the secretion of many kinds of angiogenic factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF).[1] At the same time, tumor cells also secrete antiangiogenic factors, including angiostatin and endostatin.[2,3] These factors form a network and tumor angiogenesis is dependent upon the local balance of these positive and negative regulators. Antiangiogenesis in tumors can inhibit the growth and metastases of the tumor.[4] Endostatin specially inhibits the proliferation of vessel endothelial cells stimulated by bFGF.[5,6] Systemic administration of endostatin on tumor-bearing mice keeps the primary tumors in the dormant state.[3]

Clinical trials involving angiostatin and endostatin require large quantities of bioactive recombinant proteins, which are difficult to produce. This difficulty may be resolved by tumor-specific *in vivo* delivery and expression of these antiangiogenic genes.

Hypoxic regions are characteristic of solid tumors in rodents and many types of human tumors. Oxygen partial pressure in tumors of cancer patients is 10–30 mm Hg, whereas those in normal tissues range from 24 to 66 mm Hg. The hypoxic regions of solid tumors provide some species of anaerobic bacteria such as *Bifidobacterium* and *Lactobacillus* a suitable environment to germinate and grow.[7]

Bifidobacterium bifidum and *B. longum* both can selectively germinate and grow in the hypoxic regions of solid tumors after intravenous injection.[8,9] It is currently being investigated if the genus of *Bifidobacterium* are used to achieve tumor-specific gene delivery. This provides a method and a way to transport antitumor gene expressions into tumors directly.

We selected a strain of *B. adolescentis* as a delivery system to transport endostatin gene to hypoxic tumors. *B. adolescentis* with endostatin gene were injected into the mice bearing Heps liver cancer. At 168 hours after the third injection of *B. adolescentis* with endostatin gene, *B. adolescentis* were only found in the tumors and no bacilli were found in other normal tissues. Our results indicate a strong inhibition of both the angiogenesis and the hypoxic tumor growth in the administrated group, and suggest that *B. adolescentis* might be a highly tumor-specific gene delivery vector for transporting anticancer genes into tumors in cancer gene therapy.

Materials and meth ds

Animals

BALB/c male mice (obtained from Animal Center of Nanjing Medical University, Nanjing, China) weighing 20±2 g were used in this study. Mice were fed standard rodent diet.

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ligand generated by alternative splicing of mRNAs. *Oncogene.* 1995;10:149–159.
17. Maraskovsky E, Brasel K, Teepe M, et al. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J Exp Med.* 1996;184:1953–1962.
18. Steptoe RJ, Fu F, Li W, et al. Augmentation of dendritic cells in murine organ donors by Flt3 ligand alters the balance between transplant tolerance and immunity. *J Immun.* 1997;159:5438–5491.
19. Chen K, Braun S, Lyman S, et al. Anti-tumor activity and immunotherapeutic properties of Flt3-ligand in a murine breast cancer model. *Cancer Res.* 1997;57:3511–3516.
20. Lynch DH, Andreasen A, Maraskovsky C, et al. Flt3 ligand induces tumor regression and anti-tumor immune response *in vivo. Nat Med.* 1997;3:625–631.
21. Esche C, Subbotin VM, Maliszewski C, et al. Flt3-ligand administration inhibit tumor regression growth in murine melanoma and lymphoma. *Cancer Res.* 1998;58:380–383.
22. American Cancer Society. Facts and Figures. Washington, DC: American Cancer Society; 2000.
23. Landis SH, Murray T, Bolden S, et al. Cancer statistics. *Cancer J Clin.* 1998;1:6–29.
24. Day KV, Li D, Liu S, et al. Granulocyte–macrophage colony-stimulating factor in a combination gene therapy strategy for head and neck cancer. *Laryngoscope.* 2001;111:801–806.
25. O'Malley BW, Chen SH, Schwartz MR, et al. Adenovirus-mediated gene therapy for human head and neck squamous cell cancer in a nude mouse model. *Cancer Res.* 1995;55:1080–1085.
26. Bi WL, Kim YG, Feliciano ES, et al. An HSVtk-mediated local and distant anti-tumor bystander effect in tumors of head and neck origin in athymic mice. *Cancer Gene Ther.* 1997;4:246–252.
27. Culver KW, Ram Z, Wallbridge S, et al. *In vivo* gene transfer with retroviral vector–producer cells for treatment of experimental brain tumors. *Science (Washington, DC).* 1992;256:1550–1552.
28. Ram Z, Culver KW, Walbridge S, et al. *In situ* retroviral-mediated gene transfer of the herpes simplex virus thymidine kinase gene and ganciclovir administration. *Gene Ther.* 1993;53:83–88.
29. Kianmanesh AR, Perrin H, Panis Y, et al. A "Distant" bystander effect of suicide gene therapy regression of nontransduced tumor together with a distance transduced tumor. *Hum Gene Ther.* 1997;8:1807–1814.
30. Agard C, Ligeza C, Dupas B, et al. Immune-dependent distant bystander effect after adenovirus-mediated suicide gene transfer in a rat model of liver colorectal metastasis. *Cancer Gene Ther.* 2001;8:128–136.
31. Wilson KM, Stambrook PJ, Bi WL, et al. HSV-tk gene therapy in head and neck squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg.* 1996;122:746–749.
32. Lyman SD, Brasel K, Rousseau AM, et al. The flt3 ligand: a hematopoietic stem cell factor whose activities are distinct from steel factor. *Stem Cells.* 1994;12:99–107.
33. Small D, Levenseein M, Kim E, et al. The human homolog of flt2.flt3, is selectively expressed in CD34 human bone marrow cells and is involved in proliferation of early progenitory stem cells. *Proc Natl Acad Sci.* 1994;91:459–463.
34. Rosenet O, Schiff C, Pebusque MJ, et al. Human filt3/flt2 gene: cDNA cloning and expression in hematopoietic cells. *Blood.* 1993;82:1110–1119.
35. Qiao J, Blacb ME, Caruso M. Enhanced ganciclovir killing and bystander effect of human tumor cells transduced with a retroviral vector carrying a herpes simplex virus thymidine kinase gene mutant. *Hum Gene Ther.* 2000;11:1569–1576.
36. Chen CY, Chang YN, Ryan P, et al. Effect of herpes simplex virus thymidine kinase expression levels on ganciclovir-mediated cytotoxicity and the "bystander effect". *Hum Gene Ther.* 1995;6:1467–1476.
37. Chen SH, Kosai K, Xu B, et al. Combination suicide and cytokine gene therapy for hepatic metastases of colon carcinoma: sustained anti-tumor immunity prolongs animal survival. *Cancer Res.* 1996;15:3758–3762.
38. Freund CT, Sutton MA, Dong T, et al. Adenovirus-mediated combination suicide and cytokine gene therapy for bladder cancer. *Anticancer Res.* 2000;20:1359–1365.
39. Toda M, Martuza RI, Rabkin SD. Combination suicide/cytokine gene therapy as adjuvants to a defective herpes simplex virus–based cancer vaccine. *Gene Ther.* 2001;8:332–339.
40. Mesnil M, Yamasaki H. Bystander effect in herpes simplex virus-thymidine kinase/ganciclovir cancer gene therapy: role of gap-junctional intercellular communication. *Cancer Res.* 2000;60:3989–3999.
41. Wei MX, Bougnoux P, Sacre-salem B, et al. Suicide gene therapy of chemically induce mammary tumor in rat: efficacy and distant bystander effect. *Cancer Res.* 1998;58:3529–3532.
42. Engelmann C, Heslan JM, Fabre M, et al. Importance mechanisms and limitations of the distant bystander effect in cancer gene therapy of experimental liver tumors. *Cancer Lett.* 2002;179:59–69.
43. Suan J, Michael A. The clinical problem and immunomodulation. In: Habib NA, ed. *Cancer Gene Therapy: Past Achievements and Future Challenges.* 1st ed. New York: Kluwer Academic Publishing/Plenum; 1999:292–293.
44. Liau LM, Black KL, Prins RM, et al. Treatment of intracranial gliomas with bone marrow–derived dendritic cells pulsed with tumor antigens. *J Neurosurg.* 1999;90:1115–1124.
45. Nouri-Shirazi M, Banchereau J, Bell D, et al. Dendritic cells capture killed tumor cells and present their antigens to elicit tumor-specific immune responses. *J Immunol.* 2000;165:3797–3803.
46. Braun SE, Chen K, Balzar BR, et al. Flt3 ligand antitumor activity in a murine breast cancer model: a comparison with granulocyte–macrophage colony-stimulating factor and a potential mechanism of action. *Hum Gene Ther.* 1999;10:2141–2151.
47. Von Eckardstein KL, Patt S, Zhu J, et al. Short-term neuropathological aspects of *in vivo* suicide gene transfer to the F98 rat glioblastoma using liposomal and viral vectors. *Histol Histopathol.* 2001;16:735–744.

Tumors

Heps mouse liver cancer (obtained from the Animal Center of Chinese Academy of Sciences, Shanghai, China)[10] was minced thoroughly and cancer cells were obtained at the concentration of 5×10^6 cells/mL. A total of 1×10^6 tumor cells were inoculated under the skin of the right thigh for each mouse.

Bacteria

B. adolescentis[11] was from the Chinese IFFI Microbial Center and anaerobically cultured at 37°C in TPY medium.

Plasmid construction and transformation of *B. adolescentis*[12]

A shuttle vector, pBV220,[13] containing *Amp*^{r} gene, was from the Institute of Virology, Chinese Academy of Preventive Medicine. Human endostatin gene was obtained by PCR method from human liver cDNA library (Clontech, Franklin Lakes, NJ) and inserted into pBV220 vector. Recombinant plasmid was purified by QIA prep spin miniprep kit (Qiagen, Hilden, Germany).

An overnight culture of *B. adolescentis* was inoculated in 13% milk with 0.05% cysteine–HCl and 0.5 M sucrose and anaerobically cultivated overnight at 37°C. This overnight culture was diluted 1:25 in above 13% milk buffer and cultivated at 37°C until an OD_{600} reached about 0.2. *B. adolescentis* were chilled on ice, harvested by centrifugation at 4°C, and washed twice with 0.5 M sucrose. Bacteria were resuspended in about 1/150 of the original culture volume of ice-cold 0.5 M sucrose supplemented with 1 mM ammonium citrate (pH 6.0); every 100 μL of bacteria suspension was mixed with 1.0 μg of purified pBV220/endostatin plasmid and incubated at 4°C for 3.5 hours. The above mixture was added in a precooled Gene Pulser disposable cuvette (interelectrode distance 0.2 cm; Bio-Rad, Hercules, CA). A high-voltage electric pulse (duration=1.5–3.0 milliseconds) was delivered with a Gene Pulser apparatus (Bio-Rad) by using the 25-μF capacitor and 200-Ω parallel resistance. Then the mixture was inoculated in above 13% milk buffer and cultivated at 37°C for 2.5 hours, plated on the solid TPY medium (1.5% agar) with 10% rabbit blood and containing 50 μg/mL ampicillin, and incubated at 37°C under the anaerobic condition for 72 hours.

Determination of *B. adolescentis* transfected with endostatin by PCR method

The plasmids of *B. adolescentis* transfected with endostatin gene were extracted as reported.[14] PCR was performed with the following primers: N-terminal, 5′ CCG GAA TTC ATG CAC AGC CAC CGC GAC TTC CAG CCG 3′ and C-terminal, 5′ GCC GGA TCC CTA CTT GGA GGC AGT CAT GAA GCT 3′, 94°C for 1 minutes, 60°C for 1 minutes, 72°C for 1 minutes, 30 cycles.

Expression of endostatin from *B. adolescentis* and Western blot analysis

B. adolescentis with endostatin gene were incubated at 30°C for 12 hours, diluted in about 1/50 of fresh TPY medium, and incubated at 42°C for 6 hours when OD_{600} reached 0.5–0.7. Then the bacteria were harvested by centrifugation, resuspended in SDS sample buffer, and sonicated. The sample was subjected to SDS-PAGE using 12% polyacrylamide gels, followed by Western blot analysis. Purified endostatin (Calbiochem, Darmstadt, Germany) acted as positive control and normal *B. adolescentis* as negative control. The primary polyclonal mouse antihuman endostatin antibody was diluted 200 times. The secondary antibody was a 1:50 dilution of goat antimouse IgG-HRP (Sino-American Biotechnology, Shanghai, China). The membrane was stained by DAB.

Detection of antibiotics resistance and utilization of carbon source of *B. adolescentis* with endostatin gene

Antibiotics susceptibility of *B. adolescentis* with endostatin gene was detected by disc agar diffusion method. The concentration of antibiotics was 30 μg/mL. The bacteria were plated on TYP solid medium containing different antibiotics paper discs and cultured at 37°C for 72 hours, and the diameter of inhibition rings of antibiotic was measured.

B. adolescentis were inoculated in media containing different carbon source. After weekly incubation at 37°C in anaerobic condition, they were detected by the color of indicator. Normal *B. adolescentis* were examined as control.

Treatment on tumor-bearing mice with *B. adolescentis* with pBV220/endostatin through tail vein

Before injection, the *B. adolescentis* were washed three times with 5% glucose in 0.9% NaCl and were resuspended with this buffer. A total of 0.4 mL of the above suspension was injected into the tumor-bearing mice through tail vein (1×10^8 bacilli/mouse). Injections were carried out on days 3, 6, 9, 12, and 15 after inoculation of tumor cells. Mice were divided into three groups (eight mice per group). One group was injected with endostatin-carrying *B. adolescentis*, one group was treated with normal *B. adolescentis*, and one was treated with 5% glucose in 0.9% NaCl. The animals were sacrificed on day 18 and tumors were excised and weighed. The level of inhibition of tumor growth was determined by the formula as follows:

$$\frac{\text{tumor weight of control group} - \text{tumor weight of treatment group}}{\text{tumor weight of control group}} \times 100\%.$$

Examination of location and number of *B. adolescentis* with endostatin gene after the injection

After 72 hours of inoculation, the tumor-bearing mice were injected with *B. adolescentis* carrying endostatin gene through tail vein (1×10^8 bacilli/mouse), and the mice were successively treated with the same dose of *B. adolescentis* every 72 hours. At 1, 24, 48, 96, and 168 hours after the third injection, six tumor-bearing mice were sacrificed. Under aseptic conditions, tissue samples were obtained from the lung, liver, spleen, kidney, heart and tumor. Each tissue sample (0.1 g) was placed into a homogenizer to prepare a homogenate with 1 mL of 5% glucose in 0.9% NaCl. The

homogenates were diluted and plated on the solid TPY medium (1.5% agar) with 10% rabbit blood, and incubated at 37°C under anaerobic conditions for 72 hours. Then the number of colonies per dish was counted to determine the number of viable bacteria.

Immunohistochemistry

Mice were sacrificed after 18 days of treatment as described above. Tumors were excised and fixed in 4% paraformaldehyde. Tissues were frozen, and the sections (20 μm in thickness) of the tissues were cut and mounted on glass slides. The sections were subsequently treated with 0.1 M PBS (pH 7.0) for 5 minutes and methanol mixed with 0.3% H_2O_2 for 10 minutes. The sections were blocked with 1% milk in PBS, then incubated at 37°C for 2 hours with 1:200 dilution of rat antimouse CD-31 monoclonal antibody (Pharmingen, San Diego, CA), followed by 1 hour of incubation at 37°C of 1:25 dilution of goat–antirat IgG-HRP (Kirkegaard and Perry Laboratories, Gaithersburg, CA). The sections were stained by 0.05% DAB mixed with 0.03% H_2O_2, and photographed. The positive cells were quantitated by 801 Morphologic Analysis software (Jieda, Jiangsu, China).

DNA fragmentation assay[15]

The tumor tissues were excised and homogenized thoroughly. Following centrifugation at 3000×*g* at 4°C for 5 minutes, the pellets were resuspended in a lysis buffer containing 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1% SDS, 20 μg/mL DNase-free RNase, and 200 μg/mL proteinase K. After overnight incubation at 37°C, the DNA was purified by phenol/chloroform extraction, precipitated, and resuspended in TE buffer (pH 7.4). The DNA (10 μg) was subjected to electrophoresis on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide and visualized under UV light. Electrophoresis was carried out in TE buffer (pH 8.0) at 20 V for 8 hours.

Data analysis

The data were statistical analyzed by using the Student's *t* test, and *P* values of <.05 were considered to be significant.

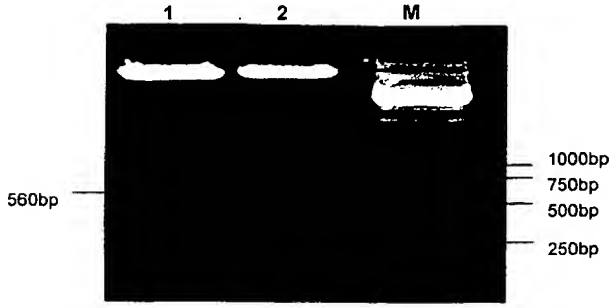

Figure 1 Molecular analysis of endostatin gene in pBV220 plasmid. pBV220/endostatin plasmid was digested by restrictive enzyme and electrophoresed in 1.0% agarose gel. A band of about 560 bp fragment was shown in pBV220/endostatin digested by *Bam*HI and *Eco*RI (**lane 1**), contrasted with no same band in pBV220 digested by the same restrictive enzyme (**lane 2**).

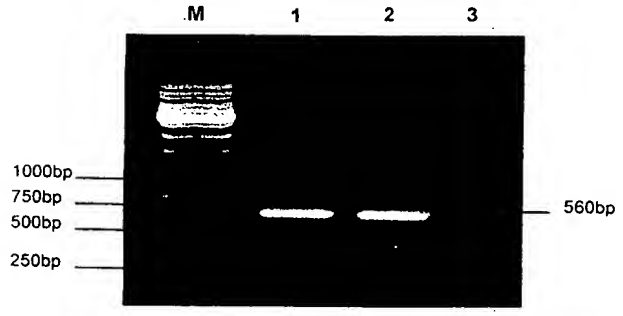

Figure 2 Identification of endostatin gene by PCR analysis. A band of 560 bp was shown from *B. adolescentis* transfected with pBV220/endostatin plasmid (**lane 2**). A parallel band was obtained by PCR amplification of endostatin from human liver cDNA library (**lane 1**). Negative result was shown from normal *B. adolescentis* (**lane 3**).

Results

Endostatin expression plasmid construction

Human endostatin gene was obtained by PCR method, and restrictive sites of *Eco*RI and *Bam*HI were inserted in the PCR primers. The pBV220/endostatin plasmid was transfected into *Escherichia coli* $DH_{5\alpha}$, and the positive clones were selected and digested with *Eco*RI and *Bam*HI. A band of about 560 bp was shown (Fig 1).

Determination of B. adolescentis with endostatin gene by PCR method

The plasmids extracted from *B. adolescentis* were amplified by PCR with above primers. A band of about 560 bp was obtained from the *B. adolescentis* with endostatin gene, and the negative result was obtained from normal *B. adolescentis* (Fig 2).

Expression of endostatin from B. adolescentis carrying pBV220/endostatin plasmid

The expression of endostatin from *B. adolescentis* was assayed by Western blot analysis using a polyclonal mouse antihuman endostatin antibody. Positive control, recombinant endostatin protein migrated at about 20 kDa. A same band about 20 kDa also was observed from transfected *B. adolescentis*. There was no positive antibody reactivity band from normal *B. adolescentis* (Fig 3).

Antibiotics resistance and carbon source utilization

Results showed that transfected *B. adolescentis* and wild *B. adolescentis* both could be killed easily by several an-

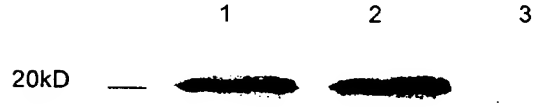

Figure 3 Western blot analysis of expression of endostatin gene. A Western blot band from *B. adolescentis* transfected with pBV220/endostatin plasmid (**lane 1**) was shown on 20 kDa parallel with the band from purified recombinant endostatin (**lane 2**), and no same band was shown from untransfected *B. adolescentis* (**lane 3**).

Table 1 The difference of antibiotic susceptibility of *B. adolescentis* carrying pBV220/endostatin plasmid (A) and wild-type *B. adolescentis* (B)

	Ceftazidime	Amoxicillin	Timethoprim	Piperacillin
A	30	28	22	33
B	29	30	21	30
	Cefradine	**Furazolidone**	**Kanamycin**	**Ciprofloxacin**
A	30	27	21	0
B	30	28	20	0
	Penicillin	**Ampicillin**	**Norvancomycin**	**Cefoperazone**
A	31	3	19	28
B	33	30	19	28

B. adolescentis were tested by disc agar diffusion method. The sensitivity of antibiotic was described by the diameter of inhibition ring (mm).

tibiotics (Table 1). Resistance of ampicillin of transfected *B. adolescentis* was due to the *Amp*^{r} gene in the recombinant plasmid. There was no difference between *B. adolescentis* with endostatin gene and normal *B. adolescentis* in carbon source utilization (Table 2).

Selective growth of B. adolescentis with pBV220/endostatin in tumor tissues

The tumor-bearing mice were intravenously injected with 1×10^8 viable transfected *B. adolescentis*; sacrificed at 1, 24, 48, 96, and 168 hours after the third injection; and examined for the presence of bacilli in tumors and several normal tissues. At 168 hours, about 1.2×10^7 bacilli/g tumor tissue were found, but no bacilli were detected in normal tissues such as the liver, spleen, kidney, and lung from the tumor-bearing mice (Fig 4). The increasing number of bacilli in tumors suggested that the transformed *B. adolescentis* germinated in the tumor tissue. In contrast, the number of *B. adolescentis* in normal tissues decreased immediately after injection, which indicated that *B. adolescentis* did not germinate in these normal tissues (Fig 5).

Table 2 The difference of utilization of carbon source of *B. adolescentis* carrying pBV220/endostatin plasmid (A) and wild-type *B. adolescentis* (B)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
A	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+	−	−
B	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+	−	−

(+) The carbon source could be utilized by *B. adolescentis*.
(−) The carbon source could not be utilized by *B. adolescentis*.
(1) Arabinose; (2) ribose; (3) xylose; (4) glucose; (5) mannose; (6) fructose; (7) sucrose; (8) starch; (9) maltose; (10) lactose; (11) cellobiose; (12) melezitose; (13) galactose; (14) raffinose; (15) sorbitol; (16) inulin; (17) salicin.

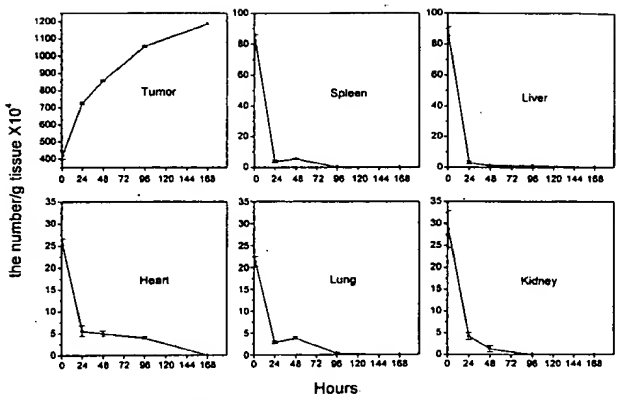

Figure 4 Organ distribution of *B. adolescentis* transfected with endostatin gene after the third administration of 1×10^8 viable bacilli into tumor-bearing mice through tail vein for each time. After 24 hours, the numbers of bacilli in spleen, liver, heart, lung, and kidney decreased significantly. After 168 hours, no bacilli were detectable in normal tissues. Each point represents mean±SD for six mice.

B. adolescentis with plasmid pBV220/endostatin inhibits the growth of primary tumors

The growth of primary tumors was potently suppressed by systemic therapy with *B. adolescentis* carrying endostatin gene. Tumor growth was inhibited by 69.9% as compared to control mice treated with 5% glucose in 0.9% NaCl. The tumor growth of mice treated by normal *B. adolescentis* was inhibited by 23.1%, compared with control group. The inhibitory role of normal *B. adolescentis* was rather weak than that of *B. adolescentis* carrying endostatin gene

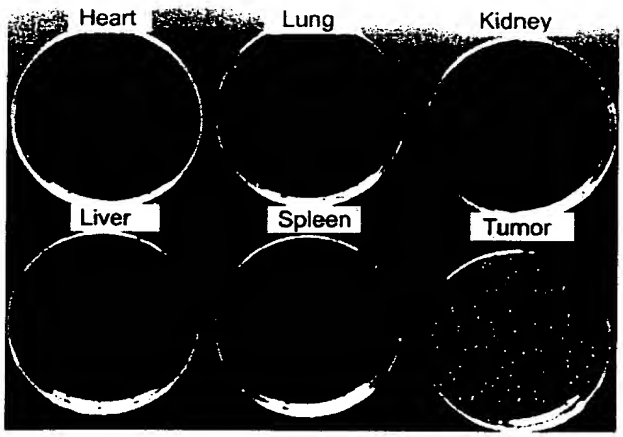

Figure 5 Comparison of the number of *B. adolescentis* carrying pBV220/endostatin plasmid in both tumors and normal tissues, after 168 hours of the third administration of 1×10^8 viable bacilli into tumor-bearing mice through tail vein for each time. The homogenate of 100 μL of sample was plated on the ampicillin-containing dishes. The homogenate of tumor tissue was diluted 1000-fold especially. After 72 hours of anaerobic cultivation, approximately 120 colonies of *B. adolescentis* carrying pBV220/endostatin plasmid were observed in the tumor, but no colonies were found in normal tissues.

($P<.01$, Fig 6). The results suggested that the role of endostatin gene was strong in the inhibition of tumor growth.

Inhibition of tumor angiogenesis by B. adolescentis with endostatin gene

Immunohistochemical analysis showed a potent inhibition of angiogenesis in the tumors treated by *B. adolescentis* with endostatin gene. Quantitative determination of vessel density was made by microscopic measurement of the brown areas (positive) at 432-fold magnification. One unit visual field encompassed 128×128 pixels. There were significantly less microvessels in the tumors of treated mice *versus* control mice after staining of the tumor tissue sections with a rat antimouse CD-31 monoclonal antibody (Fig 7). The results showed that *B. adolescentis* with endostatin

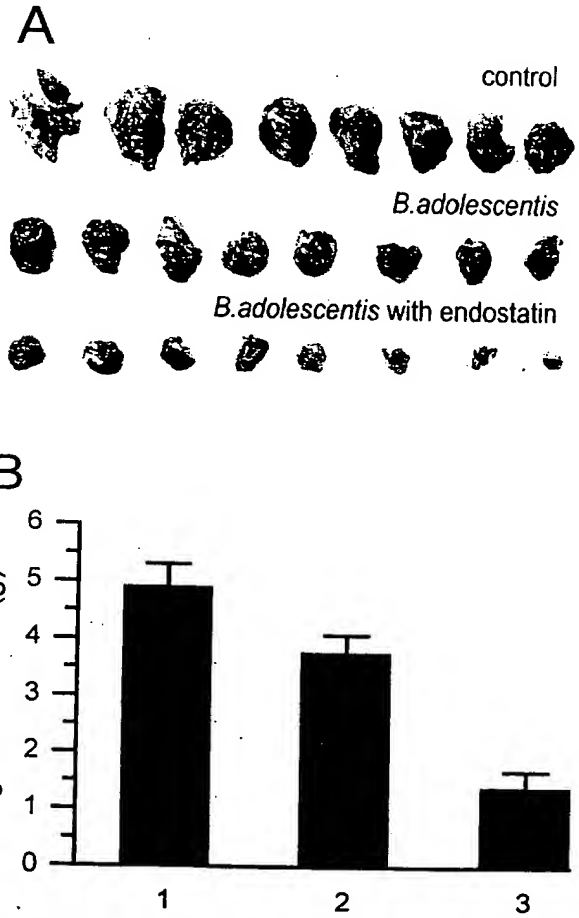

Figure 6 A: Photograph of tumors excised from representative mice treated with 5% glucose and 0.9% NaCl **(top row)**, with normal *B. adolescentis* **(middle row)**, and with *B. adolescentis* carrying endostatin gene **(bottom row)**. The volumes of tumors from three groups were much different, which indicates strong inhibition of tumor growth by endostatin-carrying *B. adolescentis*. **B:** Bar 1 was weight of tumors treated with 5% glucose and 0.9% NaCl; bar 2 was weight of tumors treated with normal *B. adolescentis*; bar 3 was weight of tumors treated with *B. adolescentis* with endostatin gene. Each bar represents the mean±SD for eight mice.

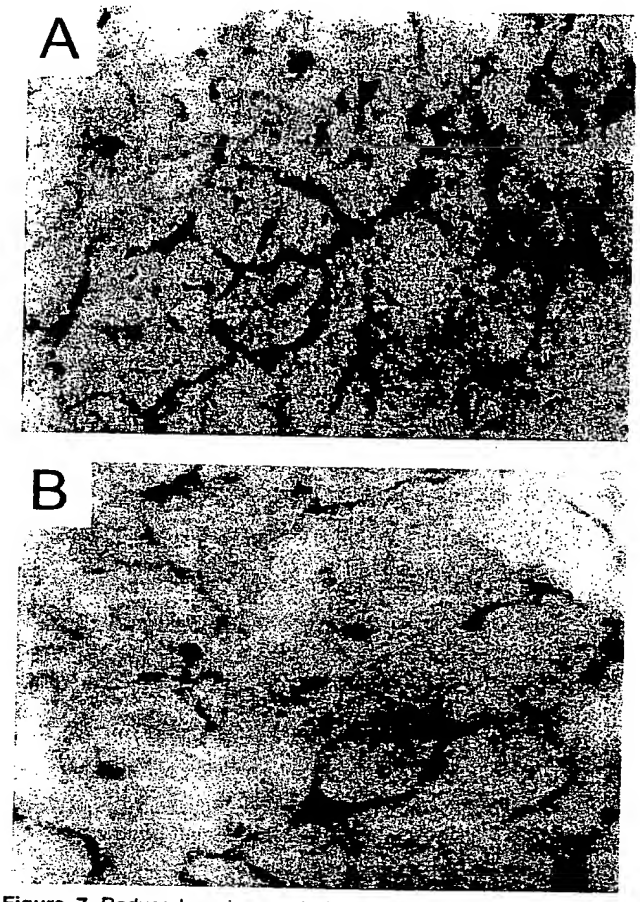

Figure 7 Reduced angiogenesis in transfected *B. adolescentis*–treated tumors. After these tumor sections were treated with CD-31 monoclonal antibody, the positive reaction cells were brown and the negative were colorless. The brown area of administrated group (**B:** tumor-bearing mice treated with *B. adolescentis* carrying endostatin gene) was 666.8±58.7 μm^2 (mean±SD) and gray scale was 50.5±8.9 (mean±SD). In the control group (**A:** treated with 5% glucose and 0.9% NaCl), the brown area was 1319.4±200.5 μm^2 (mean±SD) and gray scale was 77.0±8.3 (mean±SD). The positively stained area and gray scale of the administrated group were significantly lower ($P<.01$ and $P<.05$, respectively) than those of the control group.

gene blocked angiogenesis, but normal *B. adolescentis* did not block angiogenesis, which suggested that the inhibition of angiogenesis was caused by the introduced endostatin gene.

Induction of apoptosis in tumor cells by the introduced endostatin gene

Fragmentation of cellular DNA represents a main change in the nuclei of cells undergoing an apoptosis. Administration of *B. adolescentis* carrying endostatin gene led to the typical DNA ladder pattern in apoptotic tumor cells (Fig 8). However, no DNA fragmentation could be detected in tumor cells from mice treated with normal *B. adolescentis* or in the mice treated with 5% glucose in 0.9% NaCl. These results

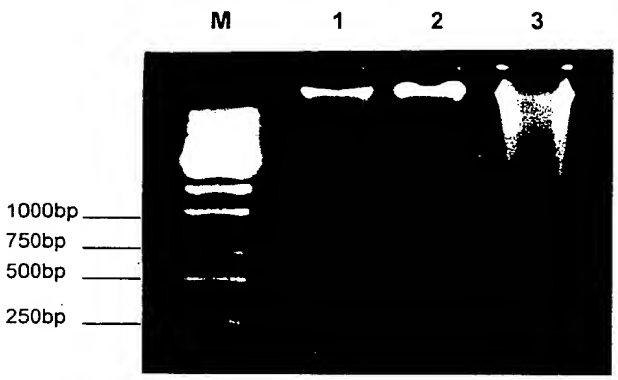

Figure 8 The introduced endostatin gene induced apoptosis in tumor cells. DNA was extracted from tumors and DNA fragmentation was identified on 1.5% agarose gel. A typical ladder pattern of apoptosis was shown in **lane 3** from the tumors of mice treated with *B. adolescentis* carrying endostatin gene. Same ladders patterns of apoptosis were not found in **lane 1** (mice treated with 5% glucose and 0.9% NaCl) and **lane 2** (mice treated with normal *B. adolescentis*).

showed that the introduced endostatin gene blocked angiogenesis accompanied by induction of apoptosis in liver tumors.

Discussion

An important obstacle for cancer gene therapy is specific gene delivery system introducing anticancer gene into target tumors. We selected a strain of *B. adolescentis* as gene delivery vector. After *B. adolescentis* with endostatin gene were intravenously injected into tumor-bearing mice, viable bacilli could be examined initially in most tissues throughout the body. However, after 96–168 hours of injection, *B. adolescentis* could be found only in tumor tissue. These data showed that *B. adolescentis* selectively germinated and proliferated in tumor tissue, and suggested that tumor tissue provided a suitable condition for *B. adolescentis* with endostatin gene to grow. In this method for cancer gene therapy, only the hypoxic regions of solid tumors were suitable for administration of *Bifidobacterium* with anticancer gene.

There are several advantages of selecting *Bifidobacterium* as gene delivery vector for cancer gene therapy: (1) *Bifidobacterium* is a domestic anaerobic bacterium in the human body that does not produce endotoxin and toxin; (2) *Bifidobacterium* increases immune response[16] and inhibits many tumor growth *in vivo* such as liver cancer, breast cancer, *etc.*;[17] (3) *Bifidobacterium* can be killed easily by antibiotics or in oxygen environment *in vitro* or *in vivo*. We confirmed that both wild-type and genetically engineered *B. adolescentis* were killed easily with kanamycin, cefoperazone, and penicillin *in vitro*; (4) when *B. adolescentis* was injected intravenously, bacilli only germinated and proliferated in solid tumor but not in other normal tissues; and (5) we demonstrated that *B. adolescentis* carrying endostatin gene specifically located in solid tumor and selectively inhibited angiogenesis and hypoxic tumor growth. These results strongly suggested that *B. adolescentis* could be used as a highly specific gene delivery vector for anticancer gene therapy.

Tumor growth and metastasis are angiogenesis-dependent. Angiogenic inducers secreted by tumor cells induce endothelial cells proliferation and migration into tumor, and form new vessels. The new intratumoral blood vessels provide nutrients and oxygen to tumor cells, and they are the way for tumor cells entering the circulation and metastasizing to distant organs. Many antiangiogenic factors detected in recent years inhibit vessel endothelial cell proliferation, induce tumor degradation and apoptosis, and limit tumor migration. Endostatin is an endogenous angiogenic inhibitor. It specifically suppresses endothelial cell proliferation, and acts as a competitor of angiogenic inducers secreted by tumor cells.[3] The inhibition of tumor growth was associated with endostatin levels in tumor model and was nearly the same in different models of solid tumors.[18] Other authors reported that endostatin showed no toxicity in all normal tissues, and that it could inhibit tumor metastasis with no drug resistance.[3]

Endostatin as an inhibitor of angiogenesis has shown an attractive new strategy in cancer gene therapy. However, poor solubility of recombinant endostatin protein and its high effective dose hampered its widespread application, although high and continuous endostatin expression can be obtained by direct induction of the endostatin gene *in vivo*. Many reports have shown that several vectors, such as viral vector,[19,20] nonviral vector, and liposome,[18] have been used in animals as gene delivery systems. However, *Bifidobacterium* as a gene therapy vector has its special characters. It can specifically reach tumor tissue by circulation and grow in hypoxic solid tumors. *Bifidobacterium* is beneficial rather than nonpathogenic for its host, immunobiologic,[16,21] anti-infective,[22] and antiaging,[23,24] and observably inhibits the growth of tumors in mice.[25] Therefore, with *Bifidobacterium* as a vector for gene therapy, its inherent role as antitumor makes it more effective in inhibition of tumor growth.

Endostatin induced DNA fragmentation in the nuclei of solid tumor cells. The fragmentation of nuclear DNA is one of the distinct morphological changes occurring in the nucleus of an apoptotic cell. However, DNA fragmentation in the nuclei of solid tumor cells treated with wild-type *B. adolescentis* was not obviously detected. These results suggested that endostatin exerted its regulatory activity and inhibition of tumor growth in transfected *B. adolescentis*.

In summary, transfected *B. adolescentis* were introduced systemically into tumor-bearing mice, and bacteria were found only in hypoxic environment of solid tumor. *B. adolescentis* could be used as a highly specific gene delivery vector in cancer gene therapy. These engineered *B. adolescentis* carrying anticancer gene specifically inhibited angiogenesis and hypoxic tumor growth, and showed a strong regulatory role on apoptosis of solid tumor cells.

Acknowledgments

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References

1. Kandel J, Bossy-Wetzel E, Radvany F, et al. Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. *Cell*. 1991;66:1095–1104.
2. O'Reilly MS, Holmgren L, Shing Y, et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell*. 1994;79:315–328.
3. O'Reilly MS, Boehm T, Shing Y, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*. 1997;88:277–285.
4. O'Reilly MS, Holmgren L, Chen C, et al. Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat Med*. 1996;2:689–692.
5. Fan YR, Xu GX, Liu XJ, et al. Construction of a recombinant human endostatin/MBP fusion protein and study on its antitumor effects *in vitro* and *in vivo*. *Chin Cancer Biother*. 2000;7:150–151.
6. Li X, Xu GX, Wang JJ. Construction of recombinant human endostatin/MBP fusion protein and its segments and their inhibition on endothelia proliferation *in vitro*. *Chin J Cell Biol*. 2001;23:91–95.
7. Dachs GU, Patterson AV, Firth JD, et al. Targeting gene expression to hypoxic tumor cells. *Nat Med*. 1997;3:515–520.
8. Kimura NT, Taniguchi S, Aoki K. Selective localization and growth of *Bifidobacterium bifidum* in mouse tumors following intravenous administration. *Cancer Res*. 1980;40:2061–2068.
9. Yazawa K, Fujimori M, Amano J, et al. *Bifidobacterium longum* as a delivery system for cancer gene therapy: selective localization and growth in hypoxic tumors. *Cancer Gene Ther*. 2000;7:269–274.
10. Lin PF. Antitumor effect of *Actinidia chinensis* polysaccharide on murine tumor. *Chin J Oncol*. 1988;10:441–444.
11. Scharek L, Hartmann L, Heinevetter L, et al. *Bifidobacterium adolescentis* modulates the specific immune response to another human gut bacterium, *Bacteroides thetaiotaomicron*, in gnotobiotic rats. *Immunobiology*. 2000;202:429–441.
12. Argnani A, Leer RJ, van Luijk N, et al. A convenient and reproducible method to genetically transform bacteria of the genus *Bifidobacterium*. *Microbiology*. 1996;42:109–114.
13. Zhang ZQ, Yao LH, Hou YD. Construction and application of a high level expression vector containing P_RP_L promotor. *Chin J Virol*. 1990;6:111–116.
14. Park MS, Lee KH, Ji GE. Isolation and characterization of two plasmids from *Bifidobacterium longum*. *Lett Appl Microbiol*. 1997;25:5–7.
15. Chen J, Jin K, Chen M, et al. Early detection of DNA strand breaks in the brain after transient focal ischemia: implication for the role of DNA damage in apoptosis and neuronal cell death. *Neurochemistry*. 1997;69:232–245.
16. Yasui H, Ohwaki M. Enhancement of immune response in Peyer's patch cells cultured with *Bifidobacterium breve*. *J Dairy Sci*. 1991;74:1187–1195.
17. Reddy BS, Rivenson A. Inhibitory effect of *Bifidobacterium longum* on colon, mammary and liver carcinogenesis induced by 2-amino-3-methylimidazo[4,5-*f*] quinoline, a food mutagen. *Cancer Res*. 1993;53:3914–3918.
18. Chen QR, Dhruv K, Sanford AS, et al. Liposomes complexed to plasmids encoding angiostatin and endostatin inhibit breast cancer in nude mice. *Cancer Res*. 1999;59:3308–3312.
19. Sauter BV, Martinet O, Zhang WJ, et al. Adenovirus-mediated gene transfer of endostatin *in vivo* results in high level of transgene expression and inhibition of tumor growth and metastases. *Proc Natl Acad Sci USA*. 2000;97:4802–4807.
20. Nguyen JT, Wu P, Clouse ME, et al. Adeno-associated virus–mediated delivery of antiangiogenic factors as an antitumor strategy. *Cancer Res*. 1998;58:5673–5677.
21. Harshamjit SG, Kay JR, Martin LC, et al. Enhancement of immunity in the elderly by dietary supplementation with the probiotic *Bifidobacterium lactis* HN019. *Am J Clin Nutr*. 2001; 74:833–839.
22. Saavedra JM, Bauman NA, Oung I, et al. Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. *Lancet*. 1994;344:1046–1049.
23. Walter P. Effects of vegetarian diets on aging and longevity. *Nat Rev*. 1997;55:S61–S65.
24. Kikuchi-Hayakawa H, Onodera N, Matsubara S, et al. Effects of soy milk and *Bifidobacterium* fermented soy milk on lipid metabolism in aged ovariectomized rats. *Biosci Biotechnol Biochem*. 1998;62:688–1692.
25. Singh J, Rivenson A, Tomita M, et al. *Bifidobacterium longum*, a lactic acid-producing intestinal bacterium inhibits colon cancer and modulates the intermediate biomarkers of colon carcinogenesis. *Carcinogenesis*. 1997;18:833–841.